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Smad-Dependent Recruitment of a Histone Deacetylase/Sin3A Complex Modulates the Bone Morphogenetic Protein-Dependent Transcriptional Repressor Activity of Nkx3.2

Dae-Won Kim and Andrew B. Lassar*

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

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We have previously shown that Nkx3.2, a transcriptional repressor that is expressed in the sclerotome and developing cartilage, can activate the chondrocyte differentiation program in somitic mesoderm in a bone morphogenetic protein (BMP)-dependent manner. In this work, we elucidate how BMP signaling modulates the transcriptional repressor activity of Nkx3.2. We have found that Nkx3.2 forms a complex, in vivo, with histone deacetylase 1 (HDAC1) and Smad1 and -4 in a BMP-dependent manner. The homeodomain and NK domain of Nkx3.2 support the interaction of this transcription factor with HDAC1 and Smad1, respectively, and both of these domains are required for the transcriptional repressor activity of Nkx3.2. Furthermore, the recruitment of an HDAC/Sin3A complex to Nkx3.2 requires that Nkx3.2 interact with Smad1 and -4. Indeed, Nkx3.2 both fails to associate with the HDAC/Sin3A complex and represses target gene transcription in a cell line lacking Smad4, but it performs these functions if exogenous Smad4 is added to these cells. While prior work has indicated that BMP-dependent Smads can support transcriptional activation, our findings indicate that BMP-dependent Smads can also potentiate transcriptional repression, depending upon the identity of the Smad-interacting transcription factor.

Bone morphogenetic protein (BMP) signals are employed during vertebrate development to pattern gene expression in all three germ layers (62, 67). Interestingly, in many cases, BMP signals can induce expression of certain batteries of genes while simultaneously repressing the expression of other batteries of genes within the same tissue or cell (23, 39, 40, 48, 69). How does BMP signaling act simultaneously to either activate or repress expression of differing target genes? Transforming growth factor β (TGF-β) superfamily members, including BMPs, induce phosphorylation of regulatory Smads, and these receptor-activated Smads consequently bind to Smad4, translocate into the nucleus, and regulate the expression of target genes (13, 43). However, since Smads can directly bind DNA with only low affinity and broad specificity, they usually interact with other promoter-specific or cell-type-specific transcription factors to achieve transcriptional specificity (44, 71). While it has been shown that interaction of Smad1 with some transcription factors can mediate BMP-dependent activation of target genes (20), it is unclear how BMP-specific Smads act to repress other transcriptional targets.

We have been studying the role that BMP signals play in directing chondrogenesis in somites, a mesodermal precursor tissue that gives rise to cartilage, skeletal muscle, tendons, and dermis. The cartilage of the axial skeleton (vertebrae and ribs) originates from the sclerotome, which is the ventral domain of the somite (25). Somites are paired blocks of mesodermal tissue symmetrically flanking the central vertebrate axis and are induced by signals from surrounding tissues to give rise to dermis, skeletal muscle, or cartilage (23). Sonic hedgehog (Shh), which is secreted by both the notochord and floor plate of the neural tube, has been shown to be required for the proper development of the sclerotome and axial cartilage formation (5). In addition to Shh, BMPs also play an important role in cartilage differentiation (28, 48). Our laboratory has previously shown that exposure of nascent somitic cells to Shh alters the response of these cells to subsequent BMP signals (48). Exposure of chick somitic explant cultures to BMP signals induces lateral plate gene expression and antagonizes sclerotomal gene expression. In contrast, after prior exposure to Shh, BMP signals now strongly promote chondrogenesis in somitic explants (48). Nkx3.2, the vertebrate homologue of *Drosophila melanogaster* Bagpipe, is induced by Shh in paraxial mesoderm, and forced expression of Nkx3.2 can promote somitic chondrogenesis in a BMP-dependent manner (49, 75). Interestingly, induction of chondrogenesis by Nkx3.2 requires both that this factor function as a transcriptional repressor and that BMP signals are present (49).

In this work, we demonstrate that Nkx3.2 is a BMP-dependent transcriptional repressor. Transcriptional repression by Nkx3.2 is modulated by a BMP-dependent association of this transcription factor with Smad1/Smad4, which in turn stabilizes the interaction of Nkx3.2 with the histone deacetylase 1 (HDAC1)/Sin3A corepressor complex. While Smad interaction has been found to augment the association of some transcription factors with coactivators such as p300/CBP (26, 54, 55), our results indicate that Smads can also mediate the association of corepressors with other transcription factors, such as Nkx3.2. Thus, interaction of transcription factors with BMP-dependent Smads can result in either transcriptional activation or repression, depending upon the identity of the transcription factor.

* Corresponding author. Mailing address: Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 240 Longwood Ave., Boston, MA 02115. Phone: (617) 432-3831. Fax: (617) 738-0516. E-mail: andrew_lassar@hms.harvard.edu.

MATERIALS AND METHODS

Tissue culture, chemical inhibitors, and antibodies. C3H10T1/2, COS-7, MDA-MB-468, and SW480.7 cells were maintained in Dulbecco's modified Eagle (DME) medium with 10% fetal calf serum (FCS). Recombinant human

BMP4 protein was the generous gift of the Genetics Institute. For in vivo HDAC inhibition, trichostatin A (TSA), a specific HDAC inhibitor (65, 74), was obtained from Calbiochem. Anti-Myc rabbit polyclonal antibody and anti-Myc monoclonal (9E10) antibody were obtained from Upstate Biotechnology. Anti-FLAG monoclonal antibody (M2) was purchased from Sigma. Purified anti-hemagglutinin (anti-HA) monoclonal antibody was obtained from Convence. Antibodies against HDACs and corepressor proteins used in the experiment shown below in Fig. 6D were purchased from Santa Cruz Biotechnology.

Plasmids and molecular cloning. 3X-NBE-pGL3P (29), pCS2-Nkx3.2-HA (29), pCS2-Nkx3.2-VP16 (29), GAL4-DBD (58), GAL4-Nkx3.2 (49), 5X-GAL4-pGL3E (12), DN-DPC4 (19), Smad6C (18), Smad7 (51), FLAG-HDAC1 (35), FLAG-Smad1 (9), and FLAG-Smad4 (9) expression constructs have all been previously described. 3X-NBE-pGL3P contains three copies of the high-affinity Nkx3.2 binding sequence (TAAGTG), which was identified by reiterative random oligonucleotide pull-down technology employing recombinant GST-Nkx3.2 (described in reference 29). pCS2-6MT-XSmad2 and pCS2-6MT-Smad4 expression constructs were generously provided by Malcolm Whitman. To create pCS2-6MT-Smad1, the Smad1 insert in the pCS2-FLAG-Smad1 construct (9) was liberated with a 5′ Klenow-filled *Sal*I site and a 3′ *Not*I overhang and ligated into the linearized 6MT-pCS2 empty vector with a 3′ Klenow-filled *Xho*I site and a 5′ *Not*I overhang. To generate a series of six-Myc-tagged (6MT) expression constructs of Nkx3.2 used in the experiments shown below in Fig. 3 and 5, the indicated coding regions were amplified from a cDNA template by PCR and inserted into the *Nco*I/*Stu*I sites of a 6MT-pCS2 expression vehicle. To create the various GAL4 fusion Nkx3.2 expression vectors used in the experiment shown in Fig. 4, the indicated inserts from pCS2-6MT-Nkx3.2 constructs (described for Fig. 3) were liberated with a 5′ Klenow-filled *Nco*I site and a 3′ *Xba*I overhang and ligated into the GAL4 fusion vector pSG424 digested with *Sma*I and *Xba*I. To construct pCS2-Nkx3.2ΔC and pCS2-Nkx3.2ΔC-VP16 used in the experiment shown below in Fig. 4, the respective inserts in SLAX13 (49) were liberated with a 5′ Klenow-filled *Nco*I site and a 3′ *Xba*I overhang and ligated into a linearized pCS2 empty vector with a 3′ Klenow-filled *Bam*HI site and a 5′ *Xba*I overhang. All the expression vehicles newly generated in this work were verified by DNA sequencing.

Transient transfection, reporter assay, and coimmunoprecipitation (co-IP). For transient transfection, FuGENE6 from Roche was used according to the manufacturer's instructions. Following the transfections, the cells were incubated under various culture conditions for different experiments as indicated. For each transfection, 100 ng of reporter construct, 200 ng of the indicated expression plasmid, and 20 ng of pRL-TK normalization plasmid were used per single well of a 12-well plate. pCS2 empty vector was used to adjust total DNA amounts where necessary. All transfection experiments were performed in duplicate, and results were normalized to the expression of the *Renilla* luciferase transfection control.

For co-IP experiments, 5 μg of the indicated expression plasmids was used per 100-mm-diameter plate. pCS2 empty vector was used to adjust total DNA amounts where necessary. Total cell extracts were prepared in co-IP buffer containing 50 mM Tris (pH 7.8), 150 mM NaCl, 1 mM EDTA, 5 mM NaF, 1 mM Na_3VO_4, 1 mM $Na_4P_2O_7$, 1.5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 10% glycerol, 0.5% NP-40, and various protease inhibitors (Complete, EDTA-free protease inhibitor cocktail; Roche). The extracts were then centrifuged for 10 min at 10,000 × *g* at 4°C, and the supernatants were used for IP assays as previously described (31).

Biotinylated oligonucleotide pull-down and HDAC activity assays. For biotinylated oligonucleotide pull-down experiments, 15 μg of pCS2 empty vector or pCS2-Nkx3.2-HA expression construct was transfected per 150-mm plate, the transfected cells were processed for nuclear extract preparation as previously described (30) and, subsequently, the final buffer composition of nuclear extracts was adjusted to 20 mM Tris (pH 7.5), 80 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 5% glycerol, 5% sucrose, and 0.1 μg of poly(dI-dC)/ml. Biotinylated Nkx3.2 binding element (NBE) or NBE-m2 oligonucleotides were synthesized by Klenow reaction after annealing two complementary oligonucleotides as previously described, except using biotin-14-dCTP instead of [^{32}P]dCTP (29). For each pull-down, 2 pmol of biotinylated oligos and 1 mg of nuclear extracts were incubated at room temperature for 20 min, and then 50 μl of streptavidin beads were added and incubated for an additional 10 min before washing with a buffer containing 10 mM Tris (pH 7.5), 50 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 5% glycerol, and 5% sucrose. The enzymatic activities of endogenous HDACs were evaluated by using a colorimetric HDAC assay with the Color de Lys system from Biomol.

In vitro protein interaction assay. For the experiment shown below in Fig. 2A, 10 μg of pCS2 empty vector or pCS2-HA-Nkx3.2 expression plasmid per 100-mm plate was transfected into COS-7 cells, and the cells were maintained for 36 h in medium containing 10% FCS. Cell lysates were prepared in lysis buffer containing 50 mM Tris (pH 7.8), 150 mM NaCl, 1 mM EDTA, 5 mM NaF, 1 mM Na_3VO_4, 1 mM $Na_4P_2O_7$, 1.5 mM $MgCl_2$, 1 mM DTT, 10% glycerol, 1% Triton X-100, and various protease inhibitors. The lysates were then centrifuged for 10 min at 10,000 × *g* at 4°C, and 5 M NaCl was added to the supernatants to adjust to a final concentration of 500 mM. Various ^{35}S-labeled Smad proteins were made by in vitro translation reactions using the TNT Quick coupled transcription-translation system from Promega. These radiolabeled Smad proteins were then incubated with immobilized Nkx3.2 in binding buffer (50 mM Tris [pH 7.8], 150 mM NaCl, 1 mM EDTA, 5 mM NaF, 1 mM Na_3VO_4, 1 mM $Na_4P_2O_7$, 1.5 mM $MgCl_2$, 1 mM DTT, 10% glycerol, 0.5% Triton X-100, and various protease inhibitors). The Nkx3.2-associated proteins were extensively washed with binding buffer, and the Smad proteins bound to Nkx3.2 were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. For the experiment shown below in Fig. 3B, pCDNA3-FLAG empty vector, pCS2-FLAG-Smad1, or pCDNA3-FLAG-HDAC1 expression plasmids were used for transfection, and the indicated pCS2-6MT-Nkx3.2 constructs were used for in vitro translation reactions.

RESULTS

BMP signals can enhance transcriptional repression by Nkx3.2. Since the induction of chondrogenesis by Nkx3.2 is dependent upon BMP signaling (49, 75), we first examined whether the transcriptional activity of Nkx3.2 can be modulated by BMP signals. To assay the transcriptional activity of Nkx3.2, we employed 3X-NBE-pGL3P, which contains three NBE sites and the simian virus 40 basal promoter 5′ to the luciferase gene (diagramed in Fig. 1A). The NBE-driven reporter was cotransfected into C3H10T1/2 cells in either the absence or presence of an Nkx3.2 expression vehicle. While cotransfection of Nkx3.2 repressed expression of the NBE-driven reporter by 3-fold in the absence of exogenous BMP administration (Fig. 1B, compare lanes 1 and 2), in the presence of exogenous BMP-4 cotransfected Nkx3.2 repressed expression of this reporter by 20-fold (Fig. 1B, compare lanes 3 and 4). Thus, BMP signals can significantly potentiate the ability of Nkx3.2 to repress transcription of genes containing Nkx3.2 binding sites.

Inhibition of the Smad pathway eliminates the transcriptional repressor activity of Nkx3.2. Smad proteins play crucial roles in transmitting BMP signals from the cell surface to the nucleus. Because the transcriptional repressor activity of Nkx3.2 can be modulated by BMP signaling, we investigated whether Smad function is required for transcriptional repression by Nkx3.2. Nkx3.2 was cotransfected with the NBE reporter in either the absence or the presence of dominant-negative Smad4 (DN-DPC4) (19). Cotransfection of DN-DPC4 blocked the ability of Nkx3.2 to repress the NBE reporter in either the absence or presence of exogenous BMP-4 administration (Fig. 1B, lanes 6 and 8). These findings indicate that Smad function is required for both a basal level of transcriptional repression by Nkx3.2 (in the absence of exogenous BMP administration) and for BMP-stimulated transcriptional repression (in the presence of exogenous BMP-4).

Since repression of the NBE reporter by Nkx3.2 requires direct binding of Nkx3.2 to the NBEs (29), it is possible that Smad proteins may modulate Nkx3.2 function by affecting either its DNA binding or transcriptional repressor activity. To clarify this issue, we determined whether BMP administration would augment the transcriptional repressor activity of Nkx3.2 when fused to the DNA binding domain of GAL4 (GAL4-Nkx3.2) and whether dominant-negative Smad reagents could

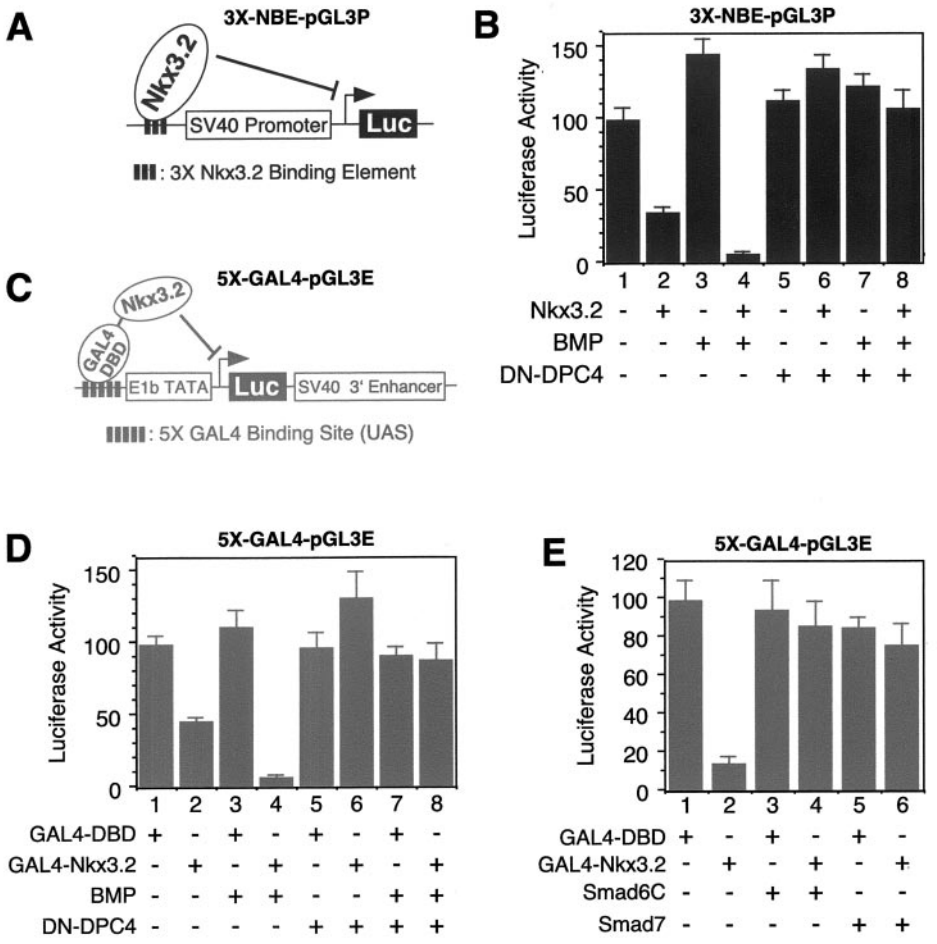

FIG. 1. The BMP-Smad pathway can modulate the transcriptional repressor activity of Nkx3.2. (A) Schematic presentation of the NBE reporter assay. (B) Nkx3.2 requires Smad function to repress expression of an NBE reporter. C3H10T1/2 cells were cotransfected with a 3X-NBE-pGL3P reporter gene plus either pCS2 empty vector (odd-numbered lanes) or pCS2-Nkx3.2 (even-numbered lanes) plus either the pCS2 control plasmid (lanes 1 to 4) or a dominant-negative Smad4 expression construct (pCS2-DN-DPC4; lanes 5 to 8). Transfected cells were grown in DME medium containing 1% FCS, and in some cases 100 ng of BMP-4/ml was included in the culture for the last 16 h of incubation (lanes 3, 4, 7, and 8). Cell extracts were subsequently processed for luciferase activity. Both this and all subsequent luciferase assays in this paper were performed in duplicate and normalized to a cotransfected reporter encoding the thymidine kinase (TK) promoter driving *Renilla* luciferase. (C) Schematic presentation of the GAL4 reporter assay. (D and E) GAL4-Nkx3.2 requires Smad function to repress expression of a GAL4 reporter. (D) COS-7 cells were cotransfected with the 5X-GAL4-pGL3E reporter construct plus expression vehicles encoding GAL4-DBD (lanes 1, 3, 5, and 7) or GAL4-Nkx3.2 (lanes 2, 4, 6, and 8) plus the pCS2 control vector (lanes 1 to 4) or pCS2-DN-DPC4 (lanes 5 to 8). Transfected cells were grown in DME medium containing 1% FCS, and in some cases 100 ng of BMP-4/ml was included in the culture for the last 16 h of incubation (lanes 3, 4, 7, and 8). Cell extracts were subsequently processed for luciferase activity. (E) COS-7 cells were cotransfected with the 5X-GAL4-pGL3E reporter construct plus expression vehicles encoding GAL4 (lanes 1, 3, and 5) or GAL4-Nkx3.2 (lanes 2, 4, and 6) plus the pCS2 control vector (lanes 1 and 2) or expression vehicles encoding Smad6C (lanes 3 and 4) or Smad7 (lanes 5 and 6). The transfected cells were grown in DME medium containing 10% FCS (which contains a BMP-like activity [data not shown]) and subsequently assayed for luciferase activity.

block transcriptional repression by this chimeric transcription factor. While GAL4-Nkx3.2 repressed transcription of a 5X-GAL4-pGL3E reporter (Fig. 1C), which contains five GAL4 binding sites and the E1b TATA box 5′ of the luciferase gene and the simian virus 40 enhancer 3′ to the gene (12), by approximately 2-fold in the absence of exogenous BMP administration (Fig. 1D, compare lanes 1 and 2), in the presence of exogenous BMP-4, GAL4-Nkx3.2 repressed expression of this reporter by 14-fold (Fig. 1D, compare lanes 3 and 4). Thus, BMP administration significantly augments the ability of the chimeric GAL4-Nkx3.2 protein to repress transcription of a GAL4 reporter, suggesting that BMP signals modulate the transcriptional repressor activity versus the NBE binding activity of Nkx3.2. Furthermore, we found that cotransfection of GAL4-Nkx3.2 with either DN-DPC4 (19), Smad6C (18), or Smad7 (21, 51), which have all been documented to block Smad signaling, could each reverse transcriptional repression of the GAL4 reporter gene by GAL4-Nkx3.2 (Fig. 1D, lanes 6 and 8, and E, lanes 4 and 6). These findings suggest that BMP signaling and Smad function modulate the transcriptional repressor activity of Nkx3.2 regardless of its DNA binding activity.

Nkx3.2 can interact with Smad proteins in vitro. Since the BMP-Smad pathway modulates transcriptional repression by

Nkx3.2, we investigated whether Nkx3.2 might form a stable complex with Smad proteins. To evaluate this, HA-Nkx3.2 was ectopically expressed in COS-7 cells and immunoprecipitated with an anti-HA antibody. Various in vitro-translated ^{35}S-labeled Smad proteins were then incubated with the immobilized Nkx3.2 in vitro, and the Smad proteins that bound to Nkx3.2 were visualized by SDS-PAGE and autoradiography. While significant levels of Smad1 bound to Nkx3.2, lesser amounts of Smad2 and Smad4 bound to this transcription factor (Fig. 2A, top panel, lanes 1 to 4). Interestingly, however, more Smad4 bound to Nkx3.2 in the presence of Smad1 (Fig. 2A, top panel, compare lanes 4 and 5). These findings suggest that Nkx3.2 may primarily bind to Smad1, which is known to specifically mediate BMP-dependent signals, and that Smad4 may be recruited to an Nkx3.2/Smad1 complex through its interaction with Smad1.

Nkx3.2 and Smads 1 and 4 can form a complex in vivo in a BMP-dependent manner. To further characterize the interaction between Nkx3.2 and Smads 1 and 4, we investigated whether an Nkx3.2–Smad1/Smad4 complex could be observed in vivo. Various combinations of expression vehicles encoding epitope-tagged Smad1, Smad4, and Nkx3.2 were cotransfected into COS-7 cells, and the in vivo association of Nkx3.2 with Smads 1 and 4 was analyzed by a co-IP assay. While only trace levels of Smad1 bound to Nkx3.2 in the absence of BMP signals (Fig. 2B, top panel, lanes 2 and 4), significantly greater levels of Smad1 bound to Nkx3.2 in the presence of BMP signals (Fig. 2B, top panel, lanes 6 and 8). Consistent with our in vitro binding assays (Fig. 2A), the association of Smad4 with Nkx3.2 required the presence of both coexpressed Smad1 and BMP-4 administration (Fig. 2B, top panel, compare lanes 3, 4, and 7 with lane 8). These results indicate that Nkx3.2 can form an in vivo complex with Smads 1 and 4 in the presence of BMP signals.

Nkx3.2 forms a BMP-dependent complex with HDAC1 in vivo. Previous studies have shown that transcriptional repression is often mediated by transcription factor-mediated recruitment of HDACs to target genes (32, 45, 52, 63, 72). Since Nkx3.2 functions as a transcriptional repressor and this activity is dependent upon BMP signaling, we wondered whether Nkx3.2 might bind to an HDAC in a BMP-dependent manner. To explore this possibility, FLAG-tagged HDAC1 and HA-tagged Nkx3.2 were ectopically expressed in COS-7 cells, and the interaction between Nkx3.2 and HDAC1 was evaluated by a co-IP assay. Interestingly, HDAC1 and Nkx3.2 form a stable complex in vivo only in the presence of BMP signals (Fig. 2C, top panel, compare lanes 3 and 4). As HDAC1 is known to be an essential constituent of a number of corepressor complexes (52, 63), these findings suggest that a BMP-dependent association of Nkx3.2 with such a corepressor complex may underlie why transcriptional repression by Nkx3.2 requires BMP signaling. To investigate if transcriptional repression by Nkx3.2 requires HDAC activity, we examined the effect of TSA, an HDAC inhibitor (65, 74), on the transcriptional repressor activity of Nkx3.2. The ability of either Nkx3.2 or GAL4-Nkx3.2 to repress transcription of either an NBE reporter or a GAL4 reporter, respectively, was completely abolished in the presence of TSA (Fig. 2D and E, compare lanes 2 and 4). Thus, HDAC activity is apparently necessary for transcriptional repression by Nkx3.2, consistent with the notion that a BMP-modulated association of Nkx3.2 with an HDAC-containing corepressor complex may mediate transcriptional repression by this transcription factor.

HDAC1 and Smad1 interact with the homeodomain and the NK domain of Nkx3.2, respectively, in vitro. Since transcriptional repression by Nkx3.2 correlates with a BMP-dependent association of this transcription factor with Smad1 and HDAC1, we decided to examine the regions of Nkx3.2 that are required to mediate these protein interactions. A series of amino- or carboxyl-terminal Nkx3.2 deletion mutants (shown schematically in Fig. 3A) were generated. Various in vitro-translated ^{35}S-labeled forms of these proteins were incubated with either immunoprecipitated Flag-Smad1 or Flag-HDAC1 that had been isolated from BMP-4 stimulated COS-7 cells. The Nkx3.2 derivatives that bound to either immobilized Flag-Smad1 or Flag-HDAC1 were visualized by autoradiography (Fig. 3B). While Nkx3.2 deletion mutants that contained the NK domain (constructs 7 to 13) were able to bind to Smad1 (Fig. 3B, gel A), Nkx3.2 deletion mutants that contained the second helix of the homeodomain (constructs 6 to 12) were able to bind to HDAC1 (Fig. 3B, gel B). These results (which are summarized in Fig. 3A) indicate that the regions of Nkx3.2 necessary to support interactions with HDAC1 or Smad1 in vitro are the homeodomain or the NK domain of Nkx3.2, respectively.

Both the homeodomain and the NK domain of Nkx3.2 are required for its repressor activity in vivo. To evaluate if the domains of Nkx3.2 that bind in vitro to either HDAC1 or Smad1 are necessary for the transcriptional repressor activity of Nkx3.2 in vivo, a series of deletion mutants of Nkx3.2 were generated in the context of a GAL4-Nkx3.2 fusion (diagrammed in Fig. 4A). Transcriptional repression by the various GAL4-Nkx3.2 mutants was analyzed by monitoring expression of the cotransfected GAL4 reporter gene (Fig. 4A and B). Because the GAL4-DNA binding domain was left intact in all these Nkx3.2 fusion proteins, we anticipated that alterations in the ability of such fusion proteins to repress transcription would reflect variable transcriptional repressor activities of the chimeric proteins, as opposed to variations in their ability to bind to target DNA sequences. While GAL4-Nkx3.2 fusions that contained both the homeodomain and the NK domain (constructs 3, 4, and 5) showed robust transcriptional repressor activity, loss of either the homeodomain (construct 6) or the NK domain (construct 2) led to a significant loss of transcriptional repressor activity. Interestingly, while a GAL4 fusion containing both the homeodomain and the NK domain (construct 5) retained a good deal of repressor activity, GAL4 fusions containing only the homeodomain (construct 7) or only the NK domain (construct 8) completely lacked transcriptional repressor activity and behaved as weak transcriptional activators. Thus, when separated from one another, neither the homeodomain nor the NK domain alone exhibits any intrinsic transcriptional repressor activity, despite the fact that they are both required for GAL4-Nkx3.2 to repress transcription. The requirement for both the NK domain and the homeodomain of Nkx3.2 to support transcriptional repression is consistent with the notion that interaction of these domains with Smad1 and an HDAC1-containing complex, respectively, is necessary for transcriptional repression by Nkx3.2.

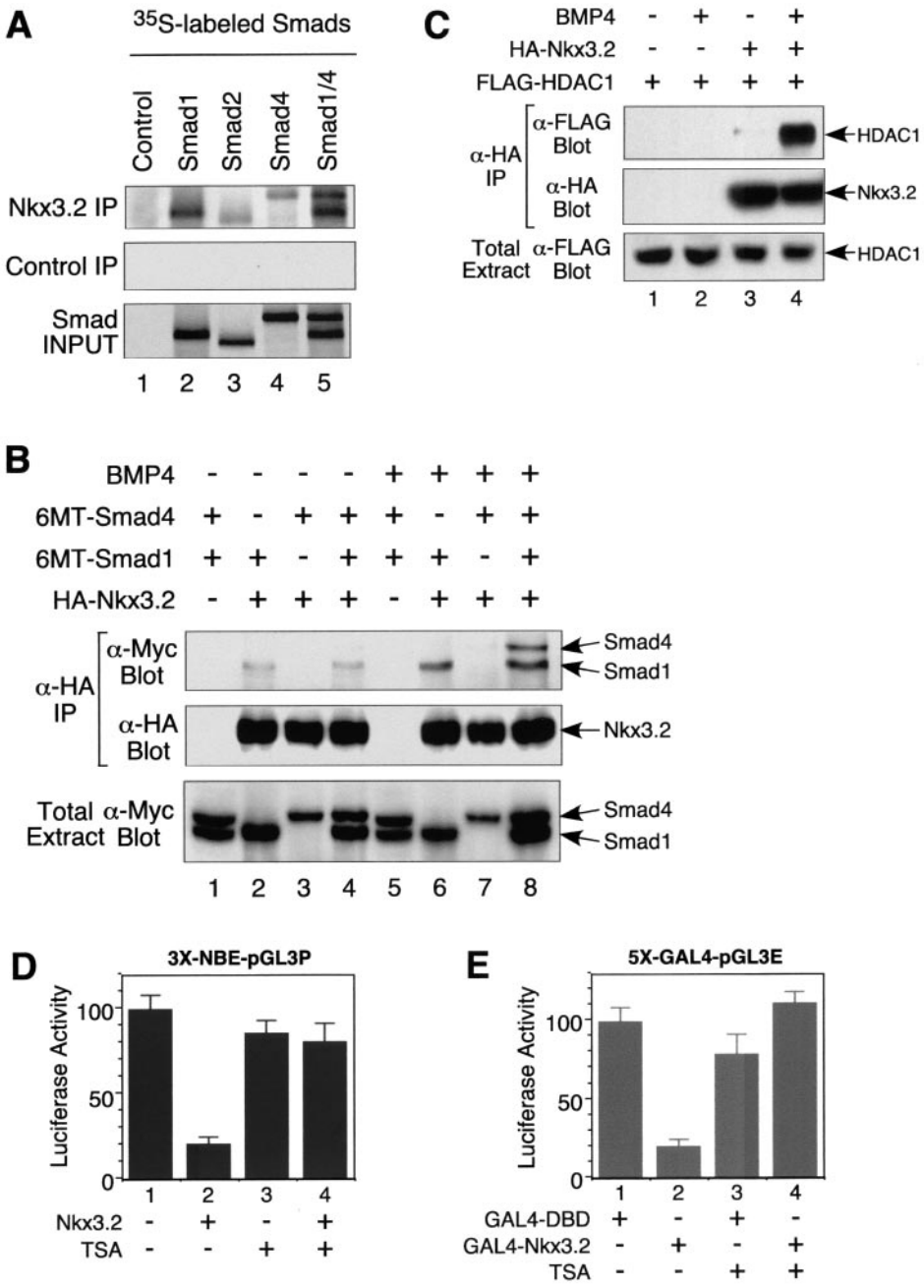

FIG. 2. Nkx3.2 associates with Smads 1 and 4 and HDAC1 in a BMP-dependent manner. (A) Smads 1 and 4 bind to Nkx3.2 in vitro. COS-7 cells were transfected with pCS2 empty vector (middle panel) or an HA-Nkx3.2 expression construct (top panel). HA-Nkx3.2 was immunoprecipitated with an anti-HA antibody, and the immunoprecipitated HA-Nkx3.2 was incubated with the indicated in vitro-translated ^{35}S-labeled Smad proteins. The bound Smad proteins were visualized by SDS-PAGE and autoradiography (top panel). The immunoprecipitates from control vehicle-transfected cells were analyzed in parallel as negative controls (middle panel). The bottom panel displays the relative input amount of each radiolabeled Smad protein. (B) Smads 1 and 4 bind to Nkx3.2 in a BMP-dependent manner in vivo. COS-7 cells were cotransfected with 6MT-pCS2-Smad1 (lanes 1, 2, 4, 5, 6, and 8) and/or 6MT-pCS2-Smad4 (lanes 1, 3, 4, 5, 7, and 8) plus pCS2 empty vector (lanes 1 and 5) or HA-Nkx3.2 expression construct (lanes 2, 3, 4, 6, 7, and 8). BMP-4 stimulation was carried out for the indicated samples (lanes 5 to 8) prior to harvesting. HA-Nkx3.2-associated proteins were immunoprecipitated with an anti-HA antibody, and the immunoprecipitates (top and middle panels) and total cell extracts (bottom panel) were Western blotted using anti-Myc (top and bottom panels) or anti-HA (middle panel) antibodies. (C) HDAC1 binds to Nkx3.2 in a BMP-dependent manner in vivo. COS-7 cells were cotransfected with expression vehicles encoding FLAG-HDAC1 (lanes 1 to 4) and HA-Nkx3.2 (lanes 3 and 4). For the indicated samples, BMP-4 was added for 30 min prior to harvesting (lanes 2 and 4). Total extracts were immunoprecipitated with an anti-HA antibody, and the immunoprecipitates (top and middle panels) and total extracts (bottom panel) were Western blotted with anti-FLAG (top and bottom panels) or anti-HA (middle panel) antibodies. (D) TSA blocks Nkx3.2 transcriptional repression of an NBE reporter. C3H10T1/2 cells were transfected with the 3X-NBE-pGL3P reporter gene plus pCS2 empty vehicle (lanes 1 and 3) or pCS2-Nkx3.2 (lanes 2 and 4). The transfected cells were grown in DME medium containing 10% FCS for 16 h, and subsequently dimethyl sulfoxide (DMSO; lanes 1 and 2) or 100 ng of TSA/ml dissolved in DMSO (lanes 3 and 4) was added to the cultures for another 12 h prior to harvesting. The relative activity of the 3X-NBE-pGL3P reporter is presented. (E) TSA blocks GAL4-Nkx3.2 transcriptional repression of a GAL4 reporter. COS-7 cells were transfected with the 5X-GAL4-pGL3E reporter construct plus expression vehicles encoding GAL4-DBD (lanes 1 and 3) or GAL4-Nkx3.2 (lanes 2 and 4). The expression of the 5X-GAL4-pGL3E reporter was evaluated in either the absence (lanes 1 and 2) or the presence (lanes 3 and 4) of TSA.

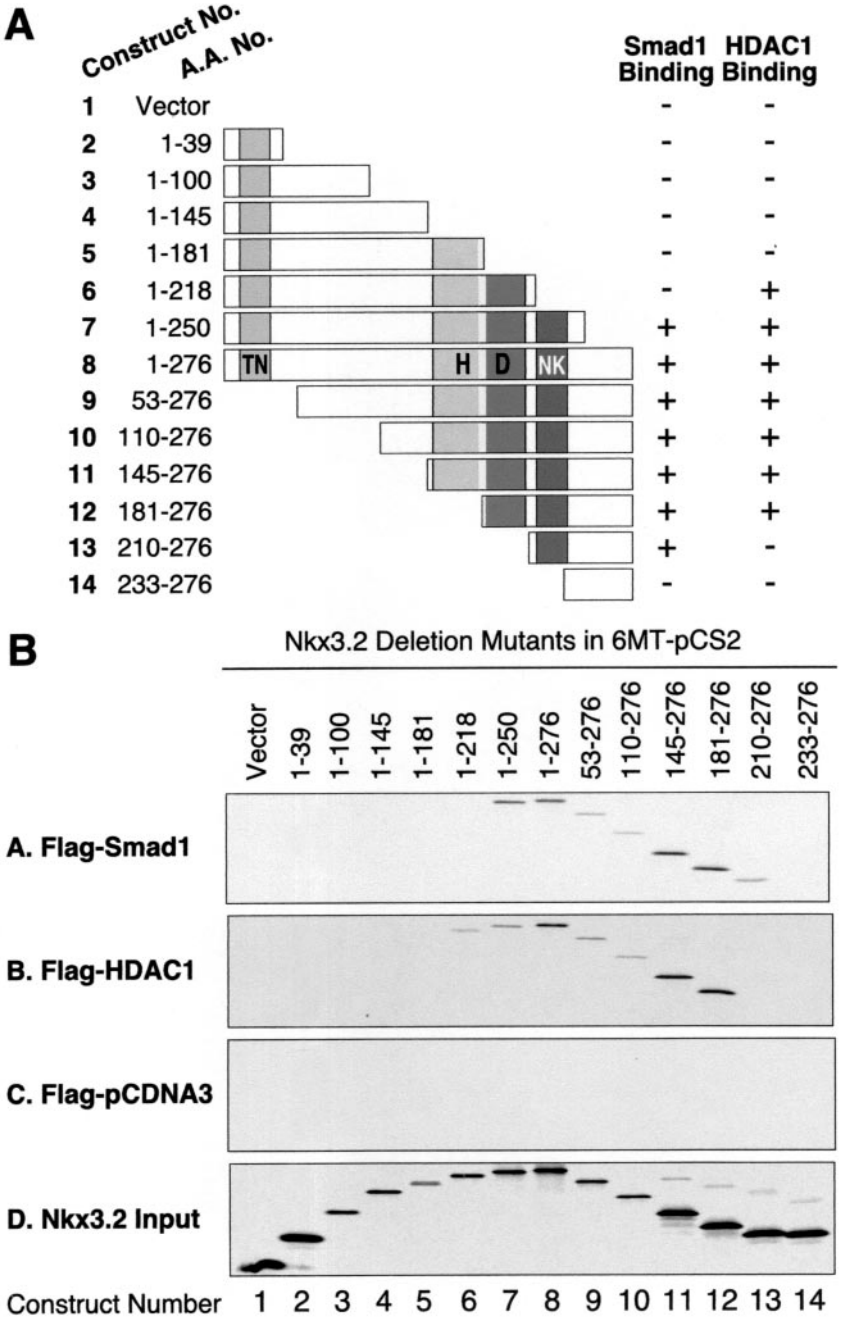

FIG. 3. Nkx3.2 binds to Smad1 and HDAC1 through its NK domain and homeodomain, respectively. (A) Diagram of various Nkx3.2 deletion mutants. A summary of Smad 1 and HDAC1 in vitro interactions with the various Nkx3.2 mutants is shown to the right of the diagram. The TN domain (amino acids 14 to 29) is conserved in some of the NK family proteins; the homeodomain (HD; amino acids 149 to 206) comprises the DNA binding domain; the NK domain (amino acids 219 to 232) is conserved in many NK family proteins. (B) COS-7 cells were transfected with expression vehicles encoding FLAG-Smad1 or FLAG-HDAC1. Two days following transfection, overexpressed FLAG-Smad1 or FLAG-HDAC1 was immunoprecipitated with an anti-FLAG antibody. The immobilized FLAG-Smad1 (A), FLAG-HDAC1 (B), or control immunoprecipitates (C) were incubated with the indicated ^{35}S-radiolabeled Nkx3.2 mutant proteins generated from in vitro translation reactions as described in Materials and Methods. The Nkx3.2 mutant proteins which bound to either immobilized Smad1 (A), HDAC1 (B), or control beads (C) were analyzed by SDS-PAGE and autoradiography. Panel D shows the relative input amount of each ^{35}S-radiolabeled Nkx3.2 protein used in the experiments.

The NK domain is necessary for BMP-dependent modulation of Nkx3.2 function. Because the carboxyl terminus of Nkx3.2, containing the NK domain, is necessary for Nkx3.2 to bind to Smad1 in vitro and to repress reporter gene transcription in vivo, we evaluated if this region of the protein was necessary for BMP-dependent modulation of Nkx3.2 activity. In contrast to Nkx3.2-WT (wild type), a derivative of Nkx3.2 lacking the carboxyl terminus (Nkx3.2ΔC; diagrammed in Fig.

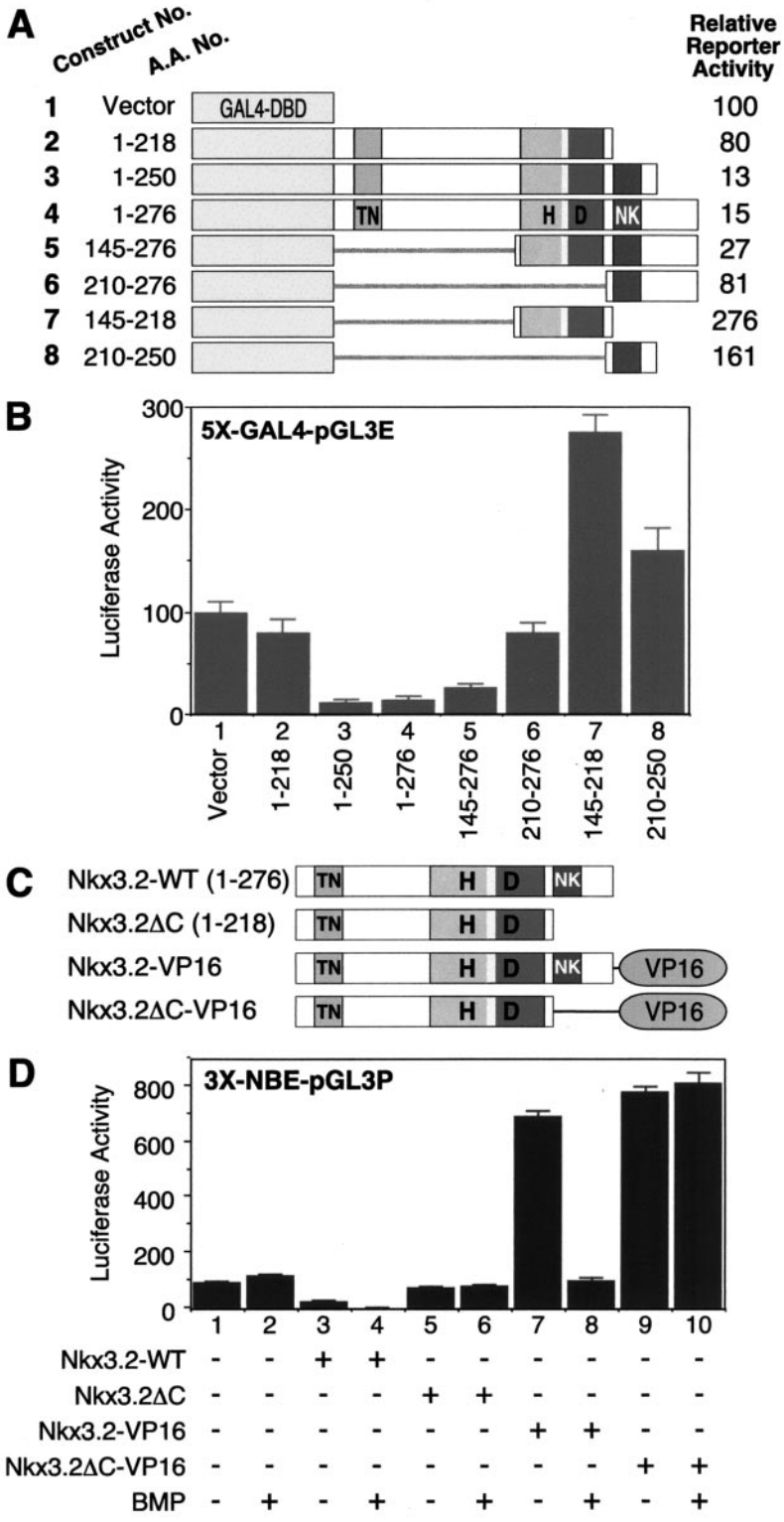

FIG. 4. Both the NK domain and homeodomain of Nkx3.2 are required for its transcriptional repressor function. (A) Schematic depicting the GAL4-DBD fused to various domains of Nkx3.2. The TN domain (amino acids 14 to 29) is conserved in some of the NK family proteins; the homeodomain (HD; amino acids 149 to 206) comprises the DNA binding domain; the NK domain (amino acids 219 to 232) is conserved in many NK family proteins. To the right of the diagram are summarized the relative expression levels of a cotransfected 5X-GAL4-pGL3E luciferase reporter, normalized to a TK-*Renilla* luciferase reporter, as displayed in panel B. (B) Both the NK domain and homeodomain of Nkx3.2 are required for its transcriptional repressor function. COS-7 cells were cotransfected with the 5X-GAL4-pGL3E reporter plus GAL4 empty vector (lane 1) or various GAL4-Nkx3.2 expression constructs (lanes 2 to 8). The relative activity of the 5X-GAL4-pGL3E reporter is displayed. (C) Diagram of various Nkx3.2 expression constructs. (D) BMP-dependent modulation of Nkx3.2 transcriptional activity is mediated by the NK domain. C3H10T1/2 cells were cotransfected with a 3X-NBE-pGL3P reporter plasmid plus either pCS2 empty vector (lanes 1 and 2) or pCS2 encoding Nkx3.2 (lanes 3 and 4), Nkx3.2ΔC (lanes 5 and 6), Nkx3.2-VP16 (lanes 7 and 8), or Nkx3.2ΔC-VP16 (lanes 9 and 10). Transfected cells were treated with BMP-4 where indicated (even-numbered lanes). Cell extracts were subsequently processed for luciferase activity.

4C) failed to repress expression of an NBE-driven reporter in response to BMP signals (Fig. 4D, compare lanes 4 and 6). In addition, we investigated if the transcriptional activity of an Nkx3.2-VP16 chimera (49), which contains the transcriptional activation domain of HSV-VP16 (57) fused to Nkx3.2 (diagrammed in Fig. 4C), was modulated by BMP signals. While administration of BMP-4 completely blocked the ability of Nkx3.2-VP16 to activate the NBE reporter (Fig. 4D, compare lanes 7 and 8), the activity of Nkx3.2ΔC-VP16 (diagrammed in Fig. 4C), which lacks the carboxyl-terminal Smad1 interaction domain, was not affected by BMP-4 administration (Fig. 4D, compare lanes 9 and 10). Together these results indicate that the carboxyl terminus of Nkx3.2, which contains the NK domain and supports interaction with Smad1 in vitro, is necessary to mediate BMP-dependent modulation of Nkx3.2 transcriptional activity.

The C-terminal domain of Nkx3.2 is necessary and sufficient to form a stable complex with Smad1 in vivo. To investigate if the carboxyl terminus of Nkx3.2 is both necessary and sufficient to mediate interaction of this protein with Smad1 in vivo, COS-7 cells were cotransfected with 6MT-Nkx3.2-WT (1-276), Nkx3.2ΔC (1-218, which lacks the carboxyl domain), or Nkx3.2-C (210-276, which encodes only the carboxyl domain) plus FLAG-Smad1; these Nkx3.2 derivatives are diagrammed in Fig. 5A. The ability of these various domains of Nkx3.2 to interact with Smad1 in vivo was analyzed by a co-IP assay. While both Nkx3.2-WT and Nkx3.2-C formed a stable complex with Smad1, Nkx3.2ΔC failed to interact with Smad1 (Fig. 5B, top panel, lanes 2 to 4). Thus, the carboxyl-terminal region of Nkx3.2, which contains the NK domain, is necessary and sufficient to mediate interaction of this transcription factor with Smad1 in vivo.

The Smad interaction domain of Nkx3.2 is required for its association with HDAC1 in vivo. Because Nkx3.2 associates with HDAC1 in vivo in a BMP-dependent manner (Fig. 2C), we wondered if this association might require simultaneous interaction of Nkx3.2 with BMP-dependent Smads. To investigate this possibility, we explored whether Nkx3.2ΔC, which lacks the Smad1 interaction domain (Fig. 5A and B) would be able to form a stable complex with HDAC1 in vivo. COS-7 cells were cotransfected with FLAG-HDAC1 and either 6MT-Nkx3.2-WT or 6MT-Nkx3.2ΔC; Nkx3.2-associated proteins were immunoprecipitated with anti-Myc antibodies. Consistent with the results shown in Fig. 2C, HDAC1 and Nkx3.2-WT formed a stable complex only in the presence of BMP signals (Fig. 5C, top panel, lane 6). In striking contrast, Nkx3.2ΔC failed to associate with HDAC1 in either the absence or presence of BMP-4 administration (Fig. 5C, top panel, lanes 3 and 4). Together with in vitro mapping of Nkx3.2 protein interaction domains (Fig. 3), these findings suggest that a BMP-inducible association of Smad1/Smad4 with the NK domain of Nkx3.2 acts to stabilize interaction of an HDAC1-containing corepressor complex with the homeodomain of Nkx3.2 in vivo. Indeed, this scenario is consistent with the necessity for both the homeodomain and NK domain of Nkx3.2 to mediate BMP-dependent transcriptional repression by this transcription factor (Fig. 4).

Association of Nkx3.2 with endogenous HDACs requires the Nkx3.2 Smad interaction domain. To evaluate whether endogenous HDACs associate with Nkx3.2 in a BMP-dependent fashion, we examined whether endogenous HDAC activity can be copurified with Nkx3.2. Epitope-tagged Nkx3.2-WT or Nkx3.2ΔC proteins were ectopically expressed in C3H10T1/2 cells, and Nkx3.2-associated proteins were immunoprecipitated from nuclear extracts made from these cells. While control immunoprecipitates did not display any appreciable HDAC activity (Fig. 5D, left panel), Nkx3.2-WT immunoprecipitates isolated from BMP-treated cells displayed significant HDAC activity (Fig. 5D, middle panel). As expected, this endogenous HDAC activity associated with immuno-purified Nkx3.2 was sensitive to TSA (Fig. 5D, middle panel). Consistent with our findings that the carboxyl terminus of Nkx3.2 is necessary for association with exogenous HDAC1 (Fig. 5C), immunoprecipitates of Nkx3.2ΔC, which lacks this domain, failed to contain any significant endogenous HDAC activity (Fig. 5D, right panel). Thus, the C-terminal Smad interaction domain is required for Nkx3.2 to recruit endogenous HDAC activity in a BMP-dependent manner.

Nkx3.2 fails to repress transcription in cell lines lacking Smad4. To further confirm that Smad signal transduction is necessary to promote the transcriptional repressor activity of Nkx3.2, we examined the activity of Nkx3.2 in cell lines lacking functional Smad4. MDA-MB-468 cells are a line of breast carcinoma cells that lack Smad4 expression (33, 59). While Smad-dependent signaling is defective in MDA-MB-468 cells, Smad signaling can be restored in these cells by ectopic Smad4 expression (56, 66). We compared the ability of Nkx3.2 to repress expression of the NBE reporter gene in either COS cells (which contain Smad4) or MDA-MB-468 cells (which lack Smad4). While cotransfection of Nkx3.2 significantly repressed expression of the NBE reporter gene in COS-7 cells (Fig. 6A, compare lanes 1 and 3), cotransfection of Nkx3.2 surprisingly augmented transcription of this reporter in MDA-MB-468 cells (Fig. 6A, compare lanes 5 and 7). Notably, the transcriptional repressor activity of Nkx3.2 on the NBE reporter was fully restored in MDA-MB-468 cells cotransfected with exogenous Smad4 (Fig. 6A, lane 8). Similar experiments were also carried out in SW480.7 cells, which are also defective in Smad signaling as a result of a Smad4 mutation (6, 17, 73), and virtually the same results were obtained (data not shown). To assess whether Smad4 is necessary for either the DNA binding or transcriptional repressor activity of Nkx3.2, we compared Nkx3.2 DNA binding activity in transfected COS-7 and MDA-MB-468 cells by evaluating the amount of HA-Nkx3.2 in extracts made from these cells that would bind to biotinylated NBE oligonucleotides. Approximately equivalent amounts of HA-Nkx3.2 bound to the biotinylated NBE oligonucleotides when isolated from either transfected COS-7 or MDA-MB-468 cells (Fig. 6B, compare lanes 3 and 7). In contrast, HA-Nkx3.2 made in either cell type failed to associate with a mutant form of the NBE site (NBE-m2) (29) which lacks crucial base pairs necessary for Nkx3.2 interaction (Fig. 6B, lanes 4 and 8). Together, these findings indicate that Nkx3.2 can bind to an NBE site but fails to repress transcription of NBE-driven reporters in the absence of functional Smad signaling.

Smad4 is necessary for Nkx3.2 to recruit HDACs to an NBE site. Because Nkx3.2 can bind to an NBE site but fails to repress transcription of an NBE reporter in the absence of Smad4 function, we wondered if Smad4 was necessary for Nkx3.2 to recruit HDAC activity to the NBE site. To evaluate

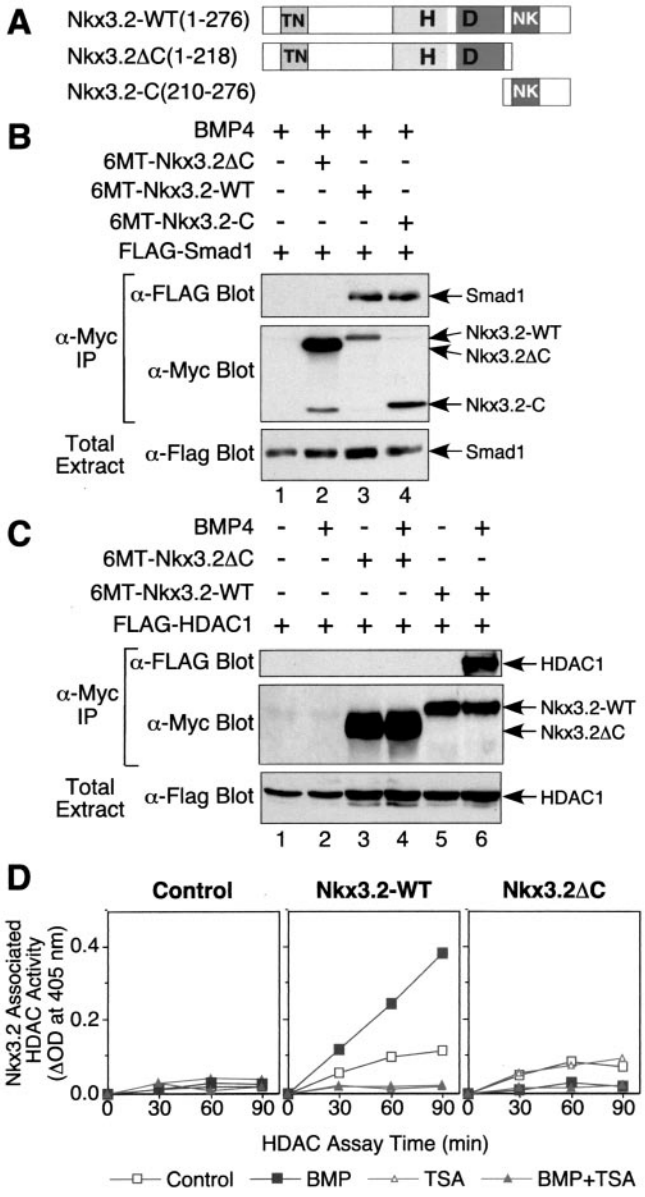

FIG. 5. The C-terminal domain of Nkx3.2 is sufficient to mediate a BMP-dependent association with Smad1 and is necessary for a BMP-dependent interaction with HDAC1 in vivo. (A) Diagram of N- or C-terminal deletion mutants of Nkx3.2. (B) The C-terminal domain of Nkx3.2 is sufficient to mediate a BMP-dependent association with Smad1 in vivo. 6MT-pCS2 empty vector (lane 1) or expression plasmids encoding 6MT-Nkx3.2ΔC (lane 2), 6MT-Nkx3.2-WT (lane 3), or 6MT-Nkx3.2-C (lane 4) were transfected into COS-7 cells along with pCS2-FLAG-Smad1 (lanes 1 to 4). The transfected cells were treated with BMP-4 for 30 min before cell lysis. 6MT-Nkx3.2-associated proteins were immunoprecipitated with an anti-Myc antibody, and the immunoprecipitates (top and middle panels) and total cell extracts (bottom panel) were Western blotted using anti-FLAG (top and bottom panels) or anti-Myc (middle panel) antibodies. (C) The C-terminal domain of Nkx3.2 is necessary to mediate a BMP-dependent interaction with HDAC1 in vivo. COS-7 cells were transfected with 6MT-pCS2 empty vector (lanes 1 and 2) or expression constructs encoding 6MT-Nkx3.2ΔC (lanes 3 and 4) or 6MT-Nkx3.2-WT (lanes 5 and 6). pCDNA3-FLAG-HDAC1 was included in all transfections (lanes 1 to 6). For indicated samples, BMP-4 stimulation was carried out for 30 min prior to harvesting (lanes 2, 4, and 6). 6MT-Nkx3.2-associated proteins were immunoprecipitated with an anti-Myc antibody, and the immunoprecipitates (top and middle panels) and total cell extracts (bottom panel) were Western blotted using anti-FLAG (top and bottom panels) or anti-Myc (middle panel) antibodies. (D) The C-terminal domain of Nkx3.2 is required for a BMP-dependent interaction with endogenous HDAC activity. C3H-10T1/2 cells were transfected with 6MT-pCS2 empty vector (left panel), pCS2-6MT-Nkx3.2-WT (middle panel), or pCS2-6MT-Nkx3.2ΔC (right panel), either grown in DME medium containing 1% FCS for the full culture period (open squares or triangles) or stimulated with BMP-4 for the last 30 min of culture (closed squares or triangles), and harvested for nuclear extract preparation. Nkx3.2 and associated proteins were immunoprecipitated employing an anti-Myc antibody, and the immunoprecipitates were assayed for HDAC activity either in the absence (open and closed squares) or presence (open and closed triangles) of TSA.

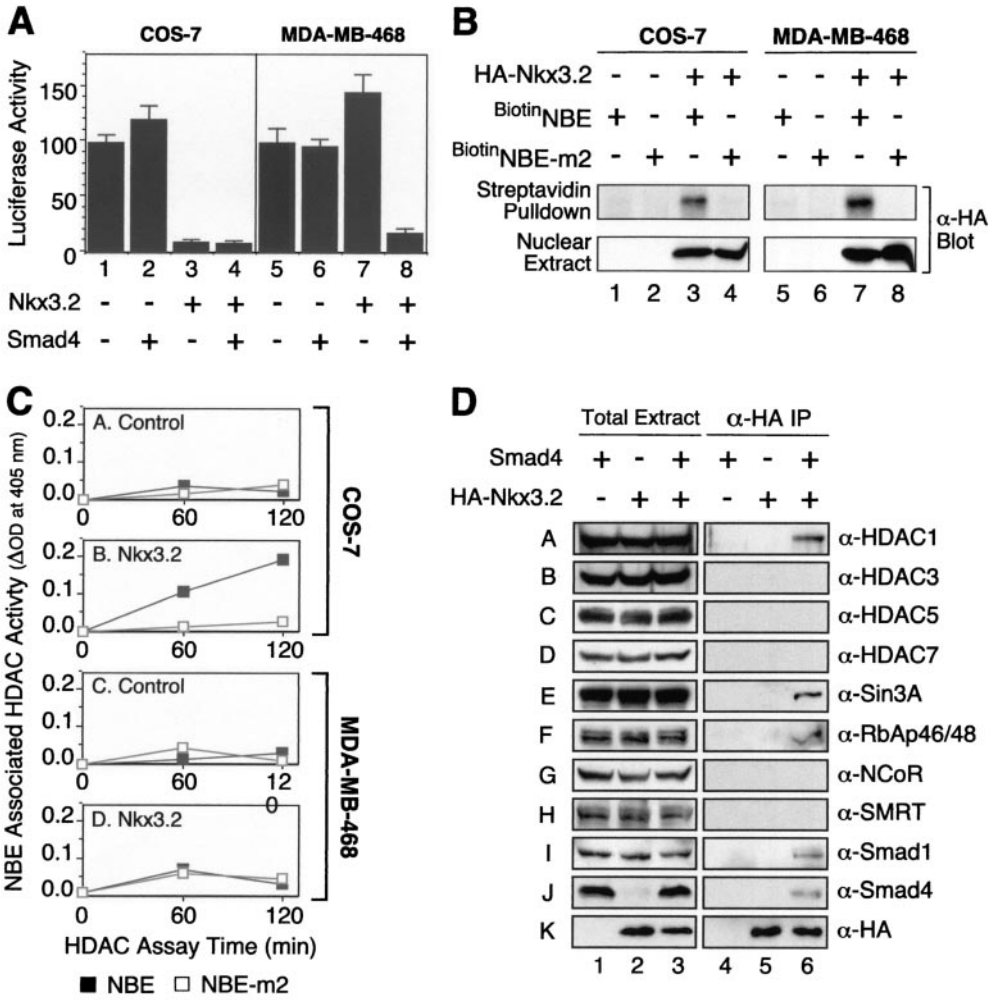

FIG. 6. Smad signaling is essential for the transcriptional repressor activity of Nkx3.2. (A) Nkx3.2 fails to repress expression of the NBE reporter in the absence of Smad4. COS-7 (Smad4-positive; lanes 1 to 4) or MDA-MB-468 (Smad4-negative; lanes 5 to 8) cells were cotransfected with a 3X-NBE-pGL3P reporter gene plus either pCS2 empty vector (lanes 1, 2, 5, and 6) or pCS2-Nkx3.2 (lanes 3, 4, 7, and 8) plus either the pCS2 control plasmid (odd-numbered lanes) or pCS2-Smad4 (even-numbered lanes). Transfected cells were grown in DME medium containing 10% FCS (which contains a high level of BMP activity [data not shown]), and the relative activity of the 3X-NBE-pGL3P reporter is displayed. (B) Nkx3.2 can bind to an NBE site in the absence of Smad4. Nuclear extracts were prepared from either COS-7 (lanes 1 to 4) or MDA-MB-468 (lanes 5 to 8) cells which had been transfected with either pCS2 empty vector (lanes 1, 2, 5, and 6) or pCS2-Nkx3.2-HA (lanes 3, 4, 7, and 8), and the respective nuclear extracts were incubated with either a biotinylated NBE oligonucleotide (odd-numbered lanes) or a biotinylated NBE-m2 oligonucleotide (which contains critical mutations in the Nkx3.2 binding site; even-numbered lanes). The NBE- or NBE-m2-associated proteins that were isolated by streptavidin agarose pull-down (top panels) or total nuclear extracts (bottom panels) were Western blotted with an anti-HA antibody. Transfected cells were grown in DME medium containing 10% FCS (which contains a high level of BMP activity [data not shown]). (C) Smad4 is necessary for Nkx3.2 to recruit an endogenous HDAC activity to an NBE site. COS-7 (A and B) or MDA-MB-468 (C and D) cells, transfected with either pCS2 empty vector (A and C) or pCS2-Nkx3.2 (B and D), were processed for nuclear extracts. Biotinylated NBE (closed squares) or biotinylated NBE-m2 (open squares) oligonucleotides were incubated with the various nuclear extracts. Following streptavidin agarose pull-down of the biotinylated oligos, associated HDAC activity was assayed. (D) Smad4 is necessary for Nkx3.2 to bind to an endogenous HDAC1/Sin3A complex. MDA-MB-468 cells were cotransfected with either pCS2 (lanes 1 and 4) or pCS2-Nkx3.2-HA (lanes 2, 3, 5, and 6) plus either pCS2 (lanes 2 and 5) or pCS2-Smad4 (lanes 1, 3, 4, and 6) expression constructs. HA-Nkx3.2 and its associated proteins were immunoprecipitated with an anti-HA antibody, and the immunoprecipitates (right panels) and total cell extracts (left panels) were Western blotted using the indicated antibodies.

this possibility, we examined if a biotinylated NBE oligonucleotide would associate with HDAC activity in extracts made from either COS-7 (Smad4-positive) or MDA-MB-468 (Smad4-negative) cells that had been transfected with Nkx3.2. A biotinylated NBE oligo bound HDAC activity in an extract made from Nkx3.2-transfected COS-7 cells, which contain Smad4 (Fig. 6C, graph B). In contrast, a biotinylated NBE-m2 oligo, which fails to associate with Nkx3.2 (29), failed to bind HDAC activity in such an extract (Fig. 6C, graph B). As opposed to these results employing COS-7 cell extracts, neither the wild-type NBE nor the NBE-m2 oligonucleotides bound appreciable HDAC activity in extracts made from Nkx3.2-

transfected MDA-MB-468 cells, which lack Smad4 (Fig. 6C, graph D). Thus, Nkx3.2 apparently requires Smad4 to recruit an endogenous HDAC activity to an Nkx3.2 DNA binding site.

Nkx3.2 interacts with an endogenous HDAC/Sin3A corepressor complex in a Smad-dependent manner. HDAC1 has been shown to be present in a variety of corepressor complexes, including the nuclear receptor-SMRT/NCoR complexes (22, 50), the Sin3A core complex (34, 77), and the NuRD complex (14). Thus, we next wanted to assess which corepressor complex might specifically associate with Nkx3.2 in a Smad-dependent fashion. To evaluate this issue, MDA-MB-468 cells were cotransfected with HA-Nkx3.2 in either the absence or the presence of exogenous Smad4. A broad range of endogenous proteins which have been found in various corepressor complexes, including HDACs 1, 3, 5, and 7 (52, 63), Sin3A (22, 50), RbAp46/48 (76), NCoR (24), and SMRT (8), were examined for their ability to interact with Nkx3.2 in vivo by a co-IP assay. The expression levels of endogenous HDACs, corepressor proteins, Smad1, and ectopic Nkx3.2 were comparable in either the presence or absence of Smad4 (Fig. 6D, gels A to K, lanes 1 to 3). Interestingly, in the presence of cotransfected Smad4, Nkx3.2 formed a stable complex in vivo with HDAC1, Sin3A, and RbAp46/48 but failed to associate with either HDACs 3, 5, and 7 or with NCoR and SMRT. Importantly, these interactions (and interaction with endogenous Smad1) could only be detected in the presence of cotransfected Smad4 (Fig. 6D, gels A, E, F, and I, lane 3). These results suggest that Nkx3.2 stably associates with the endogenous Sin3A core complex, which contains Sin3A, HDAC1, and RbAp46/48 (34, 77), and that this interaction is critically dependent on endogenous Smad4 function.

DISCUSSION

Smad signaling is required for transcriptional repression by Nkx3.2. Paraxial mesodermal cells interpret BMP signals as prochondrogenic cues only after prior exposure to Shh (48). Shh can induce expression of the transcriptional repressor, Nkx3.2, in somitic mesoderm, and forced expression of Nkx3.2 in somitic explants can substitute for Shh to promote chondrogenesis, provided that BMP signals are present (49, 75). In this report, we elucidate how BMP signals and the Smad signaling machinery promote the activity of Nkx3.2. We have demonstrated that Nkx3.2 requires a functional Smad pathway for its transcriptional repressor activity and that Nkx3.2 forms a stable complex with both Smad1/Smad4 and the HDAC/Sin3A corepressor complex in a BMP-dependent manner. The homeodomain and NK domain of Nkx3.2 mediate the interaction of this transcription factor with HDAC1 and Smad1, respectively, and both of these domains are necessary for the transcriptional repressor activity of Nkx3.2 in vivo. While Nkx3.2 can apparently bind to HDAC1 in vitro in the absence of BMP stimulation, this interaction is not manifest in vivo, unless BMP signals are supplied. Indeed, Nkx3.2 is unable to either repress transcription or bind to the HDAC/Sin3A corepressor complex in Smad4-deficient cell lines, while its DNA binding activity remains intact in such cells. However, supplementation of these mutant cells with ectopic Smad4 allows the assembly of an Nkx3.2-HDAC/Sin3A corepressor complex in the presence of BMP signals. Our findings are thus most consistent with a scenario in which BMP signals induce the association of Smads 1 and 4 with Nkx3.2 and in which this ternary complex facilitates the recruitment of the HDAC/Sin3A corepressor complex to this transcription factor in vivo (Fig. 7A). In support of this model, we have found that the NK domain containing the carboxyl terminus of Nkx3.2, which interacts with Smad1, is necessary for Nkx3.2 to form a stable complex with either exogenous HDAC1 or endogenous HDAC activity. Since the NK domain can be found in a number of NK family proteins (10, 46), it is possible that interaction of this domain with Smad proteins may allow BMP signals to similarly modulate the function of other NK family genes in addition to Nkx3.2.

In some cases modulation of the activity of a transcription factor by TGF-β family member signaling can crucially depend upon both the cellular context of the signals and the particular promoter element recognized by that transcription factor. For instance, TGF-β/Smad3 signaling converts Cbfa1/Runx2 into a transcriptional repressor in mesenchymal but not in epithelial cells, in a manner that is dependent upon the promoter sequence (2). While a molecular dissection of the requirements for BMP signals to recruit the HDAC1/Sin3A corepressor complex to Nkx3.2 was obtained in kidney (COS-7) and breast carcinoma (MDA-MB-468) cells, because Nkx3.2 acts as a BMP-dependent transcriptional repressor in both 10T1/2 mesenchymal cells (Fig. 1) and in ATDC5 chondrocytic cells (data not shown) we think it very likely that these findings are also relevant in mesenchymal cells and in chondrocytes in particular. In addition, as the ability of Nkx3.2 to induce somitic chondrogenesis requires (i) that this transcription factor function as a transcriptional repressor and (ii) that BMP signals are present (49, 75), it seems quite plausible that Smad1/Smad4 association with Nkx3.2 similarly modulates the transcriptional repressor activity of this transcription factor in somitic cells as in kidney and breast carcinoma cell lines.

Nkx3.2 associates with an HDAC/Sin3A complex distinct from the NCoR/SMRT complex. Various corepressor proteins such as NCoR or SMRT have been shown to play a critical role in the regulation of the nuclear hormone receptor family (8, 24). Recent findings have indicated that a number of nuclear hormone receptors bind to their target genes in the absence of ligand and actively repress transcription. NCoR/SMRT proteins maintain their association with such nuclear hormone receptors in the nonliganded state to bridge an interaction of the nuclear hormone receptors with a higher-order HDAC/Sin3A complex which contains Sin3A, HDACs 1, 2, 3, 4, 5, and 7, and many additional components (14, 16, 22, 45, 50). Although NCoR and SMRT have been found to interact with other transcription factors, including homeobox proteins such as Msx-1 (27, 72), we were unable to detect the association of Nkx3.2 with either NCoR/SMRT or HDACs 3, 5, and 7. Instead, we were able to demonstrate a Smad-dependent interaction of Nkx3.2 with HDAC1, Sin3A, and RbAp46/48, which are normally found as constituents of the core Sin3A complex (34, 77). While the NuRD complex contains both HDAC1 and RbAp46/48, it lacks Sin3A (14). Based on these results, we speculate that the Smad1/Smad4-Nkx3.2 complex stably associates with either the core Sin3A complex or with a novel corepressor complex in conjunction with an unidentified adaptor molecule(s) distinct from either NCoR or SMRT.

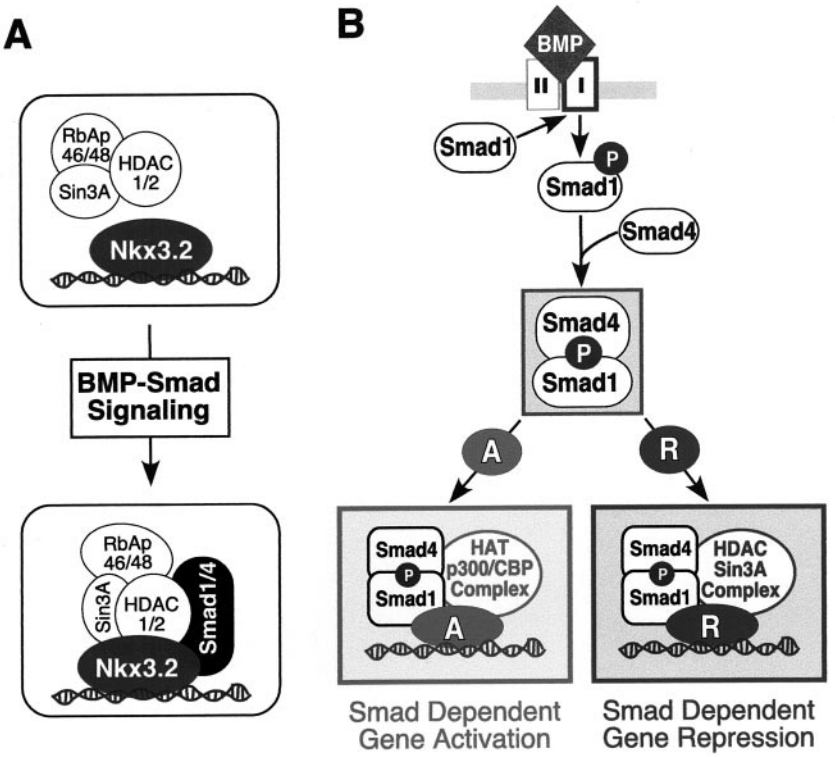

FIG. 7. The BMP-Smad pathway promotes Nkx3.2 transcriptional repression by facilitating the recruitment of an HDAC/Sin3A corepressor complex. (A) While Nkx3.2 can bind to an NBE site in the absence of Smad function, the recruitment of an HDAC/Sin3A corepressor complex to Nkx3.2 is Smad dependent. (B) BMP-specific Smads can either activate or repress target gene expression by stabilizing the association of either transcriptional coactivators or transcriptional corepressors with different transcription factors that are latent activators or repressors (schematically shown as A and R, respectively). It is not clear, however, whether Smad-dependent transcriptional repression requires the displacement of a transcriptional coactivator by a corepressor complex as indicated or the addition of a corepressor complex with the maintained association of a coactivator complex.

BMP signals allow Nkx3.2 to induce somitic chondrogenesis by blocking the expression of an antichondrogenic gene(s). Several recent studies have indicated that members of the Sox family of transcription factors play an essential role in promoting chondrogenesis (11, 36). Consistent with these findings, in the presence of exogenous BMP signals, forced expression of Nkx3.2 induces the expression of Sox9 in somites, and forced expression of Sox9 can in turn activate the chondrogenic differentiation program in a BMP-dependent manner (75). Thus, it seems most likely that Nkx3.2 induces somitic chondrogenesis by repressing the synthesis of an antichondrogenic gene(s) that blocks the expression or function of both Sox9 (3, 4, 37, 53, 60) and other prochondrogenic genes such as L-Sox5 and Sox6 (38, 61). As Nkx3.2 is induced by Shh in somitic mesoderm, it would promote chondrogenesis in derivatives of sclerotomal cells by preserving a prochondrogenic cellular environment upon exposure to secondary BMP signaling. In other words, antichondrogenic genes, which are either constitutively present or induced by BMP signals in nascent paraxial mesoderm, are either actively repressed or fail to be induced by BMP signals in Nkx3.2-expressing cells. The requirement for BMP signals to recruit the HDAC1/Sin3A corepressor complex to Nkx3.2 and thereby promote the transcriptional repressor activity of Nkx3.2 explains why BMP signals are necessary for this transcription factor to induce somitic chondrogenesis.

Smad proteins can either promote transcriptional activation or repression, depending upon the identity of their transcription partners. The Smad factors have been implicated in a broad range of biological processes. In most cases, the Smad proteins interact with various tissue-restricted transcription factors and recruit coactivators such as p300/CBP to promote cell-type-specific target gene transcription. In addition, TGF-β-dependent Smads have also been shown to interact with some transcriptional corepressors such as TGIF or Ski family members, which recruit HDACs to Smad proteins (1, 42, 64, 70) and are thought to limit the overall level of gene activation by TGF-β-dependent Smads. Interestingly, Smad-transcription factor interactions have been shown to either repress the activity of a transcription factor or modulate the activity of transcriptional repressors by multiple mechanisms. For instance, activated Smad3 can block MyoD function by interfering with MyoD/E protein dimer formation and, thus, TGF-β signaling will consequently block MyoD interaction with E-box-containing target DNA sequences (41). It has also been demonstrated that Smad3 can support TGF-β-dependent transcriptional repression of the c-*myc* promoter (7). In this instance, Smad3 was shown to associate with a p107-E2F4/5-DP1 complex in the cytoplasm in the absence of TGF-β signaling. Upon TGF-β stimulation, Smad4 binds to this complex and induces its translocation into the nucleus, where it represses expression of the

c-*myc* gene. However, as p107 and E2F4 can form a complex with HDAC1 in the absence of TGF-β signaling (15) and the transcriptional repressor activity of pRb family members is not TGF-β dependent (47, 68), the Smad3/Smad4 association with the E2F4/5-p107 complex is not essential for the intrinsic repressor activity of this transcriptional complex per se. In this instance, TGF-β signaling induces transcriptional repression of the c-*myc* gene by directing nuclear import of a cytoplasmic Smad3-E2F4/5-p107 complex. It has also been demonstrated that TGF-β-activated Smad3 induces transcriptional repression by CBFA1 (2). However, the molecular mechanism of this repression has not been elucidated.

In the present study, we demonstrated that Smads can induce transcriptional repression by stabilizing the association of a transcription factor (Nkx3.2) with the HDAC/Sin3A corepressor complex. In this case, Nkx3.2 is constitutively nuclear (data not shown), while its transcriptional repressor activity is absolutely dependent upon BMP-Smad signaling. BMP-dependent Smad proteins potentiate the transcriptional repressor activity of Nkx3.2 by facilitating Smad-dependent recruitment of an HDAC/Sin3A complex to Nkx3.2 (Fig. 7A). In contrast to Smad3-MyoD interactions, which block DNA binding of a transcriptional activator, or the Smad3/Smad4 interaction with p107-E2F4/5-DP1, which promotes nuclear translocation of a dedicated transcriptional repressor, interaction with Smad1/Smad4 modulates the transcriptional repressor activity of Nkx3.2 by regulating the association of Nkx3.2 with the HDAC/Sin3A corepressor complex. These latter observations suggest that the Smad-transcription factor interaction can be enlisted to either activate or repress target gene expression by modulating the association of differing transcription factors with either coactivators or corepressors (Fig. 7B). It is not clear, however, whether Smad-dependent transcriptional repression requires the displacement of a transcriptional coactivator by a corepressor complex as indicated in Fig. 7B or the addition of a corepressor complex with the maintained association of a coactivator complex. An outstanding question for future studies will be to elucidate why Smads recruit coactivators to some transcription factors and corepressors to others.

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